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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C07K 13/00 — A1

(11) International Publication Number: WO 93/15113

(43) International Publication Date: 5 August 1993 (05.08.93)

(21) International Application Number: PCT/US93/00358

(22) International Filing Date: 15 January 1993 (15.01.93)

(30) Priority data:
07/825,396 24 January 1992 (24.01.92) US

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(81) Designated States: AU, BB, BG, BR, CA, DK, ES, FI, HU, JP, KP, KR, LK, MG, MN, MW, NO, PL, RO, SD, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).

Published
With international search report.

(54) Title: AN IMMUNOTOXIN INCLUDING A CYTOTOXIN WITH AN UNPAIRED CYSTEINE RESIDUE IN OR NEAR ITS RECEPTOR-BINDING SITE

(57) Abstract

Disclosed are site-specifically mutated cytotoxins which have an unpaired cysteine residue in or near the cytotoxin's receptor-binding site, and which retain essentially the same receptor-binding ability and cytotoxicity as the native cytotoxins provided they are not conjugated with a binding molecule. The cytotoxins suitable for use in the invention include pseudomonas exotoxin, and diphtheria toxin, and other proteinaceous plant or bacterial toxins which have one receptor-binding site per molecule. The cytotoxins are cross-linked through the free SH group of their unpaired cysteine residues to binding molecules (including monoclonal antibodies, fragments and other ligands) to form immunotoxins, and these immunotoxins do not bind to the cell surface receptors which are bound by the native cytotoxins. However, when the cross-linker is cleaved and the binding molecule is released, the cytotoxin regains its receptor-binding ability and its cytotoxicity.

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AN IMMUNOTOXIN INCLUDING A CYTOTOXIN WITH AN UNPAIRED CYSTEINE RESIDUE IN OR NEAR ITS RECEPTOR-BINDING SITE

Field of the Invention

The invention pertains to the construction of a site-specifically mutated cytotoxin which has an unpaired cysteine residue in or near the cytotoxin's receptor-binding site, and to conjugates of these mutated cytotoxins prepared by coupling, in a cleavable manner, a specific binding molecule to the free SH group of the cysteine residue.

Background of the Invention

Since hybridoma methodologies made it possible to prepare homogenous monoclonal antibodies specific for tumor-associated cell surface antigens about fifteen years ago, the development of immunotoxins (or "magic bullets") for therapeutic applications, originally conceptualized by Paul Ehrlich at the beginning of this century, has drawn enormous interest in academia and in the biotechnology industry. Recently, the U.S. Food and Drug Administration approved the use of an anti-CD5-ricin A immunoconjugate developed by Xoma Corp. for *in vivo* therapeutic use in patients suffering from graft-vs-host disease. The same immunoconjugate is also being developed by Xoma for targeting T cells and certain B cells for suppressing the immune system in patients with rheumatoid arthritis or other autoimmune diseases. ImmunoGen Corp. is in phase II/III clinical trials of an immunotoxin for treating B cell lymphomas and leukemias, in which an anti-CD19 monoclonal antibody is conjugated with blocked ricin. Numerous other therapeutic studies and trials using immunotoxins are also being pursued.

The immunotoxin approach is especially attractive for targeting tumorous cells mainly because of the availability of extremely potent plant and bacterial protein cytotoxins, such as ricin, pseudomonas exotoxin ("PE"), and diphtheria toxin ("DT"). The amount of a cytotoxin which can be delivered to the target site by an antibody is directly related to the density of the tumor-associated antigen on the target cells. Because there typically is a low total number of antigenic molecules on a cell or in a solid tumor mass, the immunoconjugate approach is impractical if the toxin is a typical less-toxic chemotherapeutic drug, such as methotrexate, or daubicin.

In contrast, very few molecules of these bacterial and plant cytotoxins, such as ricin, PE, and DT, need to reach the cytoplasm in order to kill the target cell. These cytotoxins act by irreversibly arresting protein synthesis in eukaryotic cells. PE and DT do this by enzymatically inactivating elongation factor 2, an essential component of protein synthesis. Ricin and other plant toxins cleave a glycosidic bond in 28S ribosomal RNA, thereby destroying the ability of ribosomes to synthesize proteins. These cytotoxins have a very high activity.

The presence of functional binding domains in the cytotoxins enhances their effectiveness. Ricin, PE, and DT act by first binding to cell surface receptors. The bound cytotoxin molecules are probably endocytosed. Inside the endocytic vesicles, the enzymatic component of the toxin is somehow translocated across the vesicle membrane into the cytosol. It is thought that once in the endocytotic residues, the molecules undergo certain conformational changes that render the translocating domains of the molecules lipophilic and aid them in inserting into the membrane lipid bilayer. If this hypothesis is

correct, the conformational change is rather dramatic, because molecules like PE are hydrophilic, and there are no stretches of peptides in the polypeptide chain of PE that are hydrophobic and thus lipophilic.

Based on the principles of peptide folding and protein structure, it is likely that the structural, conformational change of the translocation domain of a cytotoxin also involves the structural change of the binding domain, since the two domains are in fact one single polypeptide chain. In other words, the binding domain of a cytotoxin contributes to the structural change of the translocation domain in order to achieve the required function for translocating the enzymatic component or the entire toxin molecule across the membrane bilayer of the endocytotic vesicles.

It is known that the ricin molecule is composed of two subunits of equal size: the A chain and the B chain. Ricin binds through its B chain to galactose-terminated oligosaccharides on the surface of cells and then transfers its A chain to the cytosol. Both PE and DT molecules are single chain polypeptides, each consisting of three discrete domains: a cell-binding, a translocating, and an elongation factor 2-inactivating enzymatic domain.

In contrast, certain other plant toxins, such as pokeweed antiviral peptide (PAP) and gelonin, have no cell-binding domain and are single-chain ribosome-inactivating proteins, similar to the A chain of ricin. These single-chain toxins are far less potent than ricin, PE, and DT because they lack cell-binding and translocating ability.

Immunotoxins constructed with these two different groups of native cytotoxins vary in their potency and specificity. Those employing ricin, PE or DT have higher potency,

but also much higher non-specific toxicity, due to their cell-binding ability. Those employing PAP or gelonin (or ricin A chain) have less nonspecific toxicity but also are less potent to the specific cell targets.

Several groups have tried to take advantage of the high potency of ricin, PE, and DT, while minimizing the non-specific toxicity of an immunotoxin which includes these cytotoxins. One approach is to decrease the affinity of ricin for galactose residues on cell surface oligosaccharides by conjugating native ricin to monoclonal antibodies and then fractioning the product by galactose affinity chromatography. The fraction that contains the immunotoxin species with impaired binding to galactose, due to the steric hindrance by the antibody's cross-linking to the ricin cell-binding site, is retained. Thorpe, P.E. et. al. *Eur. J. Biochem.* 140:63 (1984). These immunoconjugates are prepared by first modifying the antibody molecules with SH groups by reaction with 2-iminothiolane, and then conjugating the modified antibody to the ϵ-amino groups of lysyl residues of ricin using the bifunctional linking agent, N-succinimidyl 3-(2-pyridylthio) propionate (SPDP). Thorpe, P.E. and Ross, W.C.J. *Immunol. Rev.* 62:119 (1982).

Another approach for preparing a ricin-based immunotoxin in which non-specific cell-binding is diminished, is to block the two galactose-binding sites of native ricin by chemical modification with affinity ligands. Lambert, J.M. et. al. *Cancer Res.* 51:6236 (1991). The ricin molecule is reacted with reactive ligands, which are made by chemical modification of glycopeptides containing triantennary N-linked oligosaccharides derived from fetuin. Lambert, J.M. et. al. *Biochemistry* 30:3234 (1991). The sulfhydryl group is introduced at the α-amino group of the glycopeptide using 2-iminothiolane and then

capped as a mixed disulfide with 2-mercaptoethanol. The ε-amino groups of the lysyl residues of the antibody molecules are modified with succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate. This activated antibody is then reacted with the blocked ricin through the activated ligands.

Another approach for preparing immunotoxins based on PE and DT is to replace the cell-binding domain of the PE or DT polypeptide chain with a cytokine receptor or a single-chain Fv domain of an antibody molecule, using genetic engineering methods. Pastan, I. and Fitzgerald, D. *Science* 254:1173 (1991). The DNA segment of genes of transforming growth factor, interleukin-2, or interleukin-6, is spliced together with the DNA segment encoding the translocation and enzymatic domains of PE. The hybrid gene can then be expressed in *E. coli*. Similarly, genes encoding a single Fv, which comprises the variable region of the heavy chain and the light chain held together with a linking peptide, may be linked to the truncated gene of PE.

What is needed is an immunotoxin in which the cytotoxin's cell-binding site is blocked before arriving at the target site and will not bind to cells, and then the blocking agent is removed to take advantage of the high affinity of the cytotoxin for the cell-surface antigen. The unblocked cytotoxin should not be conformationally changed in a manner which affects its translocating ability. Such an immunotoxin would have very high specific potency for the target cells but reduced non-specific toxicity.

Summary of the Invention

The invention includes site-specifically mutated cytotoxins which have an unpaired

cysteine residue substitution in or near the cytotoxins' receptor-binding sites and which retain essentially the same receptor-binding ability and cytotoxicity as the native cytotoxins. These mutated cytotoxins with a steric unpaired cysteine residue are referred to as s.u.c. cytotoxins. The cytotoxins suitable for mutating to s.u.c. cytotoxins include PE, DT, and other proteinaceous plant or bacterial toxins which have one receptor-binding site per molecule. The cysteine residue will preferably replace a serine, tyrosine, asparagine, glutamine, threonine, lysine, histidine, arginine, aspartate, or glutamate residue, and the substitution will preferably not significantly affect the binding of the cytotoxins to their respective cell surface receptors.

The invention also pertains to immunotoxins in which the s.u.c. cytotoxins are linked with a cleavable cross-linker to antibodies or other binding molecules via the free SH group of the unpaired cysteine residue. While conjugated, the cytotoxins lose the ability to bind to their cell surface receptors. However, when the cross-linker is cleaved and the antibody or binding molecule is released, the cytotoxin regains its receptor-binding ability and its cytotoxicity.

The invention also includes the *in vivo* and *in vitro* applications of the immunotoxins of the invention to target and lyse the cells bearing the antigen or receptor which the binding molecules (or antibodies) recognize. The invention further includes diagnostic uses for the immunotoxins of the invention. These immunotoxins will bind to the same cell surface antigens as the binding molecules (or antibodies) which form a portion of them. Therefore, the immunotoxins can be used to determine the number or concentration of those cells which express surface antigen specifically recognized by the

binding molecules, in blood samples or cell cultures, using the stanadard assay used for cytotoxicity.

Detailed Description of the Invention

A. Cytotoxins for Site-Specific Cysteine Substitution

The protein cytotoxins which are most suitable for mutating to s.u.c. cytotoxins and for using in the immunotoxins of the invention are those: (1) which are extremely potent, killing cells at very low concentrations; (2) which have only one receptor-binding site. Two such cytotoxins are PE and DT. Mature PE is a single chain polypeptide with three discrete peptide segments, respectively responsible for binding, translocation, and ADP-ribosyltransferase activity of elongation factor 2. Mature DT contains two polypeptide chains linked by a disulfide bond. Fragment A contains the domain for ADP-ribosylation for elongation factor 2. Fragment B contains the functional sites for receptor binding and for aiding in membrane penetration by the A fragment.

B. Preparation of Cytotoxins with an Introduced Site-Specifically Unpaired Cysteine Residue Substitution in the Receptor-binding Site

For constructing the mutated cytotoxins of the invention, the cytotoxin genes are site-specifically mutated by recombinant DNA methods so that the mutated cytotoxins have an unpaired cysteine residue in or near the receptor-binding sites, such that the conjugation of a binding molecule such as an antibody, a fragment, or a factor for a receptor will block the immunotoxin's cell-binding ability. The preferred cytotoxins are single chain polypeptides containing even numbers of cysteine residues, with each pair forming a

disulfide bond. The preferred cytotoxins do not have any unpaired cysteine residues in their native form. For example, the PE molecule has 8 cysteine residues which form 4 disulfide bonds, and the DT molecule has 4 cysteine residues which form 2 disulfide bonds.

The specific pairing of the cysteine residues is determined by the 3-dimensional folding of the polypeptide chain, which is determined by the sequence of the polypeptide. The disulfide bonds are usually not exposed on the surface of the protein molecule, and their function is to hold the protein in a rigid structure to withstand the relatively harsh and variable conditions which exist outside the cytoplasm. Secreted proteins, such as cytotoxins, usually have disulfide bonds, whereas proteins which remain in the cytoplasm or on the inner surface of the plasma membrane do not have disulfide bonds.

A cysteine residue can be introduced at the receptor-binding site of a cytotoxin to provide a docking site for a binding molecule. The substitution of this residue should not affect the 3-dimensional folding of the cytotoxin molecule, or the receptor-binding and cytoxicity of the toxin. Further, the cysteine residue should be located on the surface of the protein molecule and should be accessible for cross-linking with the binding molecule.

Generally, a serine residue which is in or near a highly hydrophilic peptide stretch is most preferred for replacement with a cysteine residue. Cysteine and serine residues are structurally highly homologous. The close proximity to or the location in a hydrophilic peptide stretch will ensure that the residue will be on the surface of the protein molecule, so as to be available for cross-linking after substitution. Other preferred resides are those which are polar or charged, including asparagine, glutamine, tyrosine, histidine,

lysine, arginine, aspartate, and glutamate, provided they are in or near a peptide stretch that is hydrophilic.

The X-ray crystallographic 3-dimensional structure of some cytotoxin molecules including PE, DT, and ricin, has been determined. For those protein molecules where 3-D structure has been solved, it is possible to determine whether an amino acid residue is on the surface. However, unless the receptor-binding site is definitively determined by X-ray crystallography or by other methods, it is not possible to predict whether an amino acid residue is in or near the receptor-binding site. Where such a determination cannot be made, a suitable residue for substitution is identified by systematically determining whether the substitution of particular residues with cysteine affects the receptor binding or the biological activity of the substituted product, and whether after conjugation with a binding molecule, receptor binding is properly prevented.

A step-by-step procedure to obtain an s.u.c. cytotoxin follows.

(i) Sequencing

The first step is to determine the amino acid sequence of the cytotoxin. For most cytotoxins, including PE and DT, the sequences are available from the literature, and sequencing is not necessary. For others, sequencing can be performed by nucleotide sequencing of the cDNA clones of the mRNA of the cytotoxins. The deduced amino acid sequences can be confirmed by N-terminal amino acid sequence analysis and from a molecular weight determination of the cytotoxin proteins.

(ii) Hydrophilicity analysis

The next step is to analyze the hydrophilicity of the cytotoxin polypeptide. Several

software programs that plot the hydrophilicity (or hydropathy) in quantitative indices in relation to the linear amino acid sequence are available and can be used. One of such computer program is developed by Hopp, T.P. and Wood, K.R., and described in *Mol. Immunol.* 20:483 (1983). MicroGenie sequence analysis package distributed by Beckman Instruments, Inc. Palo, Alto, CA. provides a software program for performing hydrophilicity plots. For cytotoxins such as PE and DT, whose 3-D structure has been determined, the hydrophilicity analyses and the 3-D structure may be employed together to determine whether amino acid residues suitable for cysteine substitution are on the surface of the cytotoxin molecules.

(iii) Identifying candidate residues

The next step is to identify the hydrophilic regions in the polypeptide chain and to identify the residues in or near hydrophilic stretches best-suited for the substitution with a cysteine residue. The preferred residue for substitution is a serine residue. However, if a serine residue is not available or not suitable, a histidine, tyrosine, glutamate, aspartate, lysine, histidine, asparagine, or glutamine residue could be an alternative choice.

One first generates a number of mutant constructs (as many as ten) each having only one substitution per mutant construct. For PE, the substitutions should be made in the first domain (*i.e.* in amino acid residue Nos. 1-252), as this is believed to be the receptor-binding site, based on mutational analyses. Similarly, for DT, the substitutions should be focused on the third domain (amino acid residue Nos. 405-613), which is believed to be the receptor-binding site. Eventually, using the procedures described further below, the mutant constructs are screened to determine which have a substitution

in or near the binding site.

(iv) Gene synthesis

The next step is to synthesize the native and mutant genes. Polymerase chain reaction (PCR) can be used to construct the native cytotoxin gene. One uses oligonucleotide primers that correspond to the 5' and 3' end of the mRNA of the cytotoxin and that contain proper cloning sequences. One starts with the RNA preparation from the particular bacterial or plant specimens producing the particular cytotoxins from which cDNA is to be cloned. The cloned cDNA, after sequencing confirmation, is inserted into a plasmid, such as pUC19, for subsequent procedures. One routine laboratory procedure for site-directed mutagenesis is to start with the synthesis of oligonucleotide primers of about 25 nucleotides which contain the triplet codon of a cysteine residue in place of the triplet codon of the serine (or other) residue which is to be replaced. These primers with the installed mutations permit the synthesis of full length DNA genes with the site-directed mutations. A convenient method was developed by Kunkel, T.A., *Proc. Natl. Adac. Sci. U.S.A.*, 82:488 (1985). A step-by-step protocol with the reagents is described by Kunkel, T.A. in *Current Protocols is Molecular Biology*, Supp. 6 § 8.2.1, Eds. Ausubel, F.M. et al., Wiley Intersciences (1990). A PCR method for introducing point mutations in cloned DNA is also routinely used by many molecular biology laboratories. A step-by-step procedure is described by Cormack, B. *Current Protocols in Molecular Biology*, Supp. 15 § 8.5.1 Eds. Ausubel, F.M. et al., Wiley Intersciences (1991).

A preferred method for constructing the entire family of native genes and mutant constructs is to synthesize complete genes with a DNA synthesizer. For PE and DT

mutants, the genes encoding the receptor-binding domains, such as domain I of PE and domain III of DT, can be synthesized this way. Overlapping oligonucleotides of 60-80 nucleotides from the positive and negative strands which are complementary among the adjacent oligonucleotides at their 3' ends can be synthesized with one of the commercial DNA synthesizers, such as one from Applied Biosystems, Inc. The oligonucleotides provide both the templates and primers (mutually primed synthesis) to generate the desired sequence in one single step. After elongation is performed with T7 DNA polymerase, the segments are linked by a ligase. The oligonucleotides at the two ends of the genes are properly designed to include restriction enzyme sites, so that the synthesized genes can be inserted into the proper expression vector. The reagents to be prepared and the stepwise procedure is described by Moore, D.D., *Current Protocols in Molecular Biology*, Supp. 6 § 8.2.8, Eds. Ausubel, F.M. et al., Wiley Intersciences (1990). This method is attractive because it easily allows construction of the large number of site-directed mutations needed to make the various mutant constructs. All of the oligonucleotides, except the one with the specific mutation, may be shared for the individual constructs. Complete synthesized genes, such as interferon, have been made with these methods. *See* Edge, M.D. et al. *Interferon 7*, Ed. Gresser, I pp. 2-46 (Academic Press, London, 1986).

(v) Expression

The next step is to express the wild type and the mutated sets of cDNA in a eukaryotic or prokaryotic expression system, thus producing the native cytotoxin and the mutant cytotoxin, and then to purify the cytotoxins to produce sufficient amounts of each. Cytotoxins, such as PE and DT, which are derived from bacteria, can be expressed in the

host bacteria. Thus, PE genes may be expressed in *Pseudomonas aeruginosa* and DT genes in *Corynebacterium diphtheriae*. When an *E. coli* expression system is used, the expressed cytotoxin proteins need to be solubilized, reduced to unfold the polypeptide chain, and allowed to renature to form the most favorable 3-dimensional structure. A preferred system is the FLAG Biosystem kit, offered by International Biotechnologies of Kodak (new Haven, CT). This system also contains the reagents for the detection and purification of the non-fused protein.

(vi) Conjugation

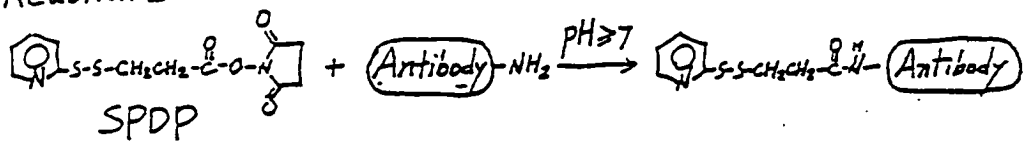
The purified native cytotoxin molecules must be tested for receptor-binding and cytotoxicity. The various mutant cytotoxins are also tested for these properties before and after the conjugation with binding molecules. A preferred binding molecule for conjugating with the purified native and mutant cytotoxins is the antibody IgG, or its $F(ab')_2$, or Fab fragment. An example of a preferred antibody for conjugation is the monoclonal antibody anti-CD5, which is specific for human T cells and for a subpopulation of B cells. The purposes of the present step are to determine whether: (1) the introduced cysteine residue is accessible for conjugation; and (2) the conjugation blocks the receptor-binding and the cytotoxicity of the cytotoxin.

The preferred cross-linking agents for linking the cytotoxins to the binding molecules are reversible disulfide formation agents. An example is N-succinimidyl 3-(2-pyridylthio) propionate (SPDP, available from Pierce Chemical Co., Rockford, IL). A procedure for preparing the antibody-toxin conjugates is described by Cumber, J.A. et al *Methods in Enzymol.* 112:207 (1985). However, in this referenced study, the sulfhydryl

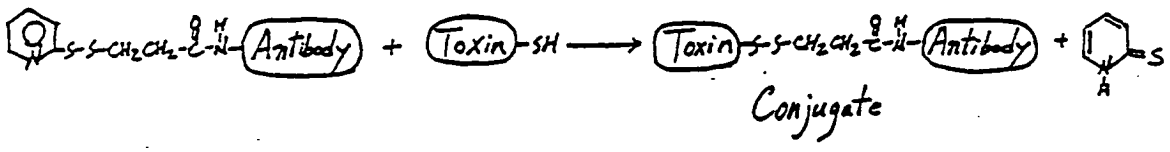
groups of the toxin molecule were introduced by the reaction of 2-iminothiolane (Traut's reagent). The introduction of SH groups with Traut's reagent creates heterogeneous products. In the present invention, the SH groups are introduced into the cytotoxin by genetic engineering methods, and the resulting s.u.c. cytotoxin is homogeneous in terms of the number and location of the SH groups.

The conjugation reaction can be summarized by the steps shown below.

Reaction 1



Reaction 2



Another preferred cross-linking agent is 4-succinimidyloxycarbonyl-α-methyl-α-(2-pyridyldithio) toluene (SMPT). Because of the bulky groups next to the disulfide bond between the cytotoxins and the binding molecule in the conjugate, these immunotoxins are more stable and not as easily reduced as those constructed with SPDP.

Before performing the conjugation reaction, however, the first step is to create a free SH group on the cytotoxin. The free SH group of an unpaired cysteine residue,

however, may be coupled to other sulfhydryl group-containing metabolites during biosynthesis. It must first be reduced, preferably under mild reducing conditions, to free it from such metabolites. Mild reducing conditions do not reduce the disulfide bonds buried inside the molecular backbone of the cytotoxin, and thus allow the cytotoxin's structure to be maintained.

After reduction, the reducing agent is removed by gel filtration or ion exchange chromatography. The treated cytotoxin is then reacted with the binding molecules, which have been previously modified with the cross-linker.

The native cytotoxin likely will not conjugate with the binding molecules, as the native cytotoxin usually does not have any accessible, unpaired cysteine residues. However, for those native cytotoxin which do have accessible unpaired cysteine residues, they can also be conjugated to the binding molecules by the procedure described above. Thereafter, they can be analyzed for receptor binding/biological activity as described immediately below, to determine whether they are s.u.c. cytotoxins. If this analysis reveals that they are not s.u.c. cytotoxins, then the unpaired cysteine residue may be replaced by a serine residue (to ensure that it does not conjugate with the binding molecules), and another residue at another location can be replaced with a cysteine residue.

This substitution of a serine for a cysteine should not affect the receptor binding or biological activity. The subsequent conjugation reaction(s) will only link the binding molecules at the one unpaired cysteine residue, and not elsewhere.

(vii) Receptor binding/biological activity

For analyzing and comparing the receptor-binding and biological activity of the native and mutant cytotoxins, and the binding molecule-conjugated mutant cytotoxins, they are tested, with a standard laboratory procedure, on cell lines which are labeled with ^{51}Cr. Biddison, W.E. *Current Protocols in Immunology*, Vol. 1, § 717.1 Eds. Coligan, J.E. et al. Wiley Intersciences (1991). The specific release of ^{51}Cr from the lysed cells indicates binding and cytotoxicity. Alternatively, the cells may be incubated with [^{3}H]-thymidine, and the specific decrease of [^{3}H]-thymidine incorporation into DNA compared to controls will also indicate the binding and toxicity of the tested products, using a standard laboratory procedure. Kruisbeek, A.M. *Current Protocols in Immunology*, Vol. 1, § 3.12.1 Eds. Coligan, J.E. et al. Wiley Intersciences (1991).

An example of a human cell line suitable for targeting with the immunotoxin is a T cell line, such as CEM, expressing CD5. In this experimental system, an anti-CD5 monoclonal antibody is the binding molecule. These monoclonal antibodies are conjugated with mutant cytotoxin molecules with particular cysteine residue substitutions, which have substantially the same receptor-binding and biological activity as the native cytotoxins and when conjugated to the antibodies yield immunotoxins which are specifically toxic to cells expressing the target antigen but not to cells without the target antigen.

C. Example: The Preparation of S.U.C. Pseudomonus Exotoxin

The cDNA gene for PE has been cloned and sequenced. The cDNA has also been expressed in *E. coli* for the production of biologically active PE. Gary, G.L. et. al. *Proc.*

Natl. Acad. Sci. U.S.A. 81:2645 (1984). The X-ray cystallographic structure of PE at 3-Angstrom resolution has been determined, and the hydrophilicity plot of PE has also been made. Allured, V.S. et. al. *Proc. Natl. Acad. Sci. U.S.A.* 83:1320 (1986). The functional domains of the PE molecule responsible for cell-binding, translocation, and enzymatic, toxic activity has also been determined. Hwang, J. et. al. *Cell* 48:129 (1987). In this last study, it was shown that domain I, amino acid residue Nos. 1-252, is involved in binding to the cell surface receptor.

PE has eight cysteine residues forming four disulfide bonds. Using a hydrophilicity analysis program provided by MicroGenie, which adopts the principles of Hopp, T.P. and Wood, K.R. *Mol. Immunol.* 20:483 (1983), a hydrophilicity plot of the peptide segment Nos. 1-275 is made (not shown). The plot indicates regions or peptide segments of relatively high hydrophilicity. Using the criteria discussed above, the amino acid residues selected for site-directed mutagenesis (*i.e.*, for substitution with cysteine residues) are: lysine No. 20, serine No. 25, serine No. 88, serine No. 96, serine No. 158, arginine No. 182, serine No. 188, serine No. 192, lysine No. 223, and serine No. 245 (creating 10 mutant constructs in total).

The preferred method for preparing the native PE gene and the ten mutant genes of PE is to construct the gene segments for the first domain with the oligonucleotide synthesis method described above. The gene segment for the second and third domains, which are not varied among the various constructs, are synthesized by PCR. The gene segments are ligated and inserted into expression vectors for expression as described in Section B above.

D. Application of S.U.C. Cytotoxins for Preparing Improved Immunotoxins

The preferred binding molecules for use in constructing the immunotoxins of the invention are monoclonal antibodies, or $F(ab')_2$, or Fab fragments, specific for tumor associated antigens on the surface of target cells. They may also be specific for tissue or cell-type-specific cell surface antigens. The monoclonal antibodies may be human or murine antibodies, or chimerized, or CDR-grafted human antibodies. The chimerization or humanization of the IgG antibody, $F(ab')_2$, and Fab fragments enhances their suitability for *in vivo* application.

The binding molecules may also be natural or genetically altered ligands, including interleukin-2, interleukin-6, or transforming growth factor, which bind to the corresponding cell surface receptors. It is known that certain tumors probably arise because of imbalance of growth factors, and that they express high concentrations of growth factor receptors on the cell surface. Pastan, I. and Fitzgerald, D. *Science* 254:1173 (1991).

The immunotoxins of the invention can be used as improved diagnostic reagents to determine the presence, in a blood sample or a cell culture, of those subsets of cells which express the surface antigen recognized by the immunotoxin's binding molecule portion. The immunotoxins, due to their potency, will lyse cells expressing the recognized surface antigen(s) at a relatively low density, compared with what a conventional immunotoxin can recognize and lyse. A conventional cytotoxicity assay, such as those relying on ^{51}Cr release or [^{3}H]-thymidine incorporation, described in Section B(vii) above, may be used.

It should be understood that the terms, expressions and examples herein are exemplary only and not limiting, and those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. All such equivalents are intended to be encompassed by the following claims.

SEQUENCE LISTING

(1) General Information:
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(ii) Title of Invention: An Immunotoxin Including a Cytotoxin with an Unpaired Cysteine Residue in or Near Its Receptor-Binding Site
(iii) Number of Sequences: 2
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(v) Computer Readable Form:
(A) Medium Type: Diskette, 3.5 inch
(B) Computer: IBM PS/2
(C) Operating System: DOS 3.30
(D) Software: Wordperfect 5.1
(vi) Current application data:
(A) Application Number:
(B) Filing Date:
(C) Classification:
(vii) Prior Application Data:
(A) Application Number:
(B) Filing Date:
(viii) Attorney/Agent Information:
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(B) Registration Number: 31,211
(C) Reference/Docket Number: TNX92-1-PCT
(ix) Telecommunication Information:
(A) Telephone: (713) 664-2288
(B) Telefax: (713) 664-8914
(2) Information for SEQ ID NO:1:
(i) Sequence Characteristics:
(A) Length: 613 amino acids
(B) Type: amino acid
(D) Topology: linear
(xi) Sequence Description: SEQ ID NO:1:

```
Ala Glu Glu Ala Phe Asp Leu Trp Asn Glu Cys Ala Lys Ala Cys
 5                  10                  15

Val Leu Asp Leu Lys Asp Gly Val Arg Ser Ser Arg Met Ser Val
            20                  25                  30
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Asp Pro Ala Ile Ala Asp Thr Asn Gly Gln Gly Val Leu His Tyr
35 40 45

Ser Met Val Leu Glu Gly Gly Asn Asp Ala Leu Lys Leu Ala Ile
50 55 60

Asp Asn Ala Leu Ser Ile Thr Ser Asp Gly Leu Thr Ile Arg Leu
65 70 75

Glu Gly Gly Val Glu Pro Asn Lys Pro Val Arg Tyr Ser Tyr Thr
80 85 90

Arg Gln Ala Arg Gly Ser Trp Ser Leu Asn Trp Leu Val Pro Ile
95 100 105

Gly His Glu Lys Pro Ser Asn Ile Lys Val Phe Ile His Glu Leu
110 115 120

Asn Ala Gly Asn Gln Leu Ser His Met Ser Pro Ile Tyr Thr Ile
125 130 135

Glu Met Gly Asp Glu Leu Leu Ala Lys Leu Ala Arg Asp Ala Thr
140 145 150

Phe Phe Val Arg Ala His Glu Ser Asn Glu Met Gln Pro Thr Leu
155 160 165

Ala Ile Ser His Ala Gly Val Ser Val Val Met Ala Gln Thr Gln
170 175 180

Pro Arg Arg Glu Lys Arg Trp Ser Glu Trp Ala Ser Gly Lys Val
185 190 195

Leu Cys Leu Leu Asp Pro Leu Asp Gly Val Tyr Asn Tyr Leu Ala
200 205 210

Gln Gln Arg Cys Asn Leu Asp Asp Thr Trp Glu Gly Lys Ile Tyr
215 220 225

Arg Val Leu Ala Gly Asn Pro Ala Lys His Asp Leu Asp Ile Lys
230 235 240

Pro Thr Val Ile Ser His Arg Leu His Phe Pro Glu Gly Gly Ser
245 250 255

Leu Ala Ala Leu Thr Ala His Gln Ala Cys His Leu Pro Leu Glu
260 265 270

Thr Phe Thr Arg His Arg Gln Pro Arg Gly Trp Glu Gln Leu Glu
275 280 285

Gln Cys Gly Tyr Pro Val Gln Arg Leu Val Ala Leu Tyr Leu Ala
290 295 300

Ala Arg Leu Ser Trp Asn Gln Val Asp Gln Val Ile Arg Asn Ala
305 310 315

Leu Ala Ser Pro Gly Ser Gly Gly Asp Leu Gly Glu Ala Ile Arg
320 325 330

Glu Gln Pro Glu Gln Ala Arg Leu Ala Leu Thr Leu Ala Ala Ala
335 340 345

Glu Ser Glu Arg Phe Val Arg Gln Gly Thr Gly Asn Asp Glu Ala
350 355 360

Gly Ala Ala Asn Ala Asp Val Val Ser Leu Thr Cys Pro Val Ala
365 370 375

Ala Gly Glu Cys Ala Gly Pro Ala Asp Ser Gly Asp Ala Leu Leu
380 385 390

Glu Arg Asn Tyr Pro Thr Gly Ala Glu Pne Leu Gly Asp Gly Gly
395 400 405

Asp Val Ser Phe Ser Thr Arg Gly Thr Gln Asn Trp Thr Val Glu
410 415 420

Arg Leu Leu Gln Ala His Arg Gln Leu Glu Glu Arg Gly Tyr Val
425 430 435

Phe Val Gly Tyr His Gly Thr Phe Leu Glu Ala Ala Gln Ser Ile
440 445 450

Val Phe Gly Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala Ile
455 460 465

Trp Arg Gly Pne Tyr Ile Ala Gly Asp Pro Ala Leu Ala Tyr Gly
470 475 480

Tyr Ala Gln Asp Gln Glu Pro Asp Ala Arg Gly Arg Ile Arg Asn
485 490 495

Gly Ala Leu Leu Arg Val Tyr Val Pro Arg Ser Ser Leu Pro Gly
500 505 510

Phe Tyr Arg Thr Ser Leu Thr Leu Ala Ala Pro Glu Ala Ala Gly
515 520 525

Glu Val Glu Arg Leu Ile Gly His Pro Leu Pro Leu Arg Leu Asp
530 535 540

Ala Ile Thr Gly Pro Glu Glu Glu Gly Gly Arg Leu Glu Thr Ile
545 550 555

Leu Gly Trp Pro Leu Ala Glu Arg Thr Val Val Ile Pro Ser Ala
560 565 570

Ile Pro Thr Asp Pro Arg Asn Val Gly Gly Asp Leu Asp Pro Ser
575 580 585

Ser Ile Pro Asp Lys Glu Gln Ala Ile Ser Ala Leu Pro Asp Tyr
590 595 600

Ala Ser Gln Pro Gly Lys Pro Pro Arg Glu Asp Leu Lys
605 610 613

(2) Information for SEQ ID NO:2:
(i) Sequence Characteristics:
(A) Length: 535 amino acids
(B) Type: amino acid
(D) Topology: linear
(xi) Sequence Description: SEQ ID NO:2:

Gly Ala Asp Asp Val Val Asp Ser Ser Lys Ser Phe Val Met Glu
5 10 15

Asn Phe Ser Ser Tyr His Gly Thr Lys Pro Gly Tyr Val Asp Ser
20 25 30

Ile Gln Lys Gly Ile Gln Lys Pro Lys Ser Gly Thr Gln Gly Asn
35 40 45

Tyr Asp Asp Asp Trp Lys Gly Phe Tyr Ser Thr Asp Asn Lys Tyr
50 55 60

Asp Ala Ala Gly Tyr Ser Val Asp Asn Glu Asn Pro Leu Ser Gly
65 70 75

Lys Ala Gly Gly Val Val Lys Val Thr Tyr Pro Gly Leu Thr Lys
80 85 90

Val Leu Ala Leu Lys Val Asp Asn Ala Glu Thr Ile Lys Lys Glu
95 100 105

Leu Gly Leu Ser Leu Thr Glu Pro Leu Met Glu Gla Val Gly Thr
110 115 120

Glu Glu Phe Ile Lys Arg Phe Gly Asp Gly Ala Ser Arg Val Val
125 130 135

Leu Ser Leu Pro Phe Ala Glu Gly Ser Ser Ser Val Glu Tyr Ile

140 145 150

Asa Asa Trp Glu Glu Ala Lys Ala Leu Ser Val Glu Leu Glu Ile
155 160 165

Asn Phe Glu Thr Arg Gly Lys Arg Gly Gln Asp Ala Met Tyr Glu
170 175 180

Tyr Met Ala Gln Ala Cys Ala Gly Asn Arg Val Arg Arg Ser Val
185 190 195

Gly Ser Ser Leu Ser Cys Ile Asn Leu Asp Trp Asp Val Ile Arg
200 205 210

Asp Lys Thr Lys Thr Lys Ile Glu Ser Leu Lys Glu His Gly Pro
215 220 225

Ile Lys Asa Lys Net Ser Glu Ser Pro Asa Lys Thr Val Ser Glu
230 235 240

Glu Lys Ala Lys Gln Tyr Leu Glu Glu Phe His Gln Thr Ala Leu
245 250 255

Glu His Pro Glu Leu Ser Glu Leu Lys Thr Val Thr Gly Thr Asn
260 265 270

Pro Val Phe Ala Gly Ala Asn Tyr Ala Ala Trp Ala Val Asn Val
275 280 285

Ala Gln Val Ile Asp Ser Glu Thr Ala Asp Asn Leu Glu Lys Thr
290 295 300

Thr Ala Ala Leu Ser Ile Leu Pro Gly Ile Gly Ser Val Met Gly
305 310 315

Ile Ala Asp Gly Ala Val His His Asn Thr Glu Glu Ile Val Ala
320 325 330

Gln Ser Ile Ala Leu Ser Ser Leu Net Val Ala Gln Ala Ile Pro
335 340 345

Leu Val Gly Glu Leu Val Asp Ile Gly Phe Ala Ala Tyr Asn Phe
350 355 360

Val Glu Ser Ile Ile Asn Leu Phe Gln Val Val His Asn Ser Tyr
365 370 375

Asn Arg Pro Ala Tyr Ser Pro Gly His Lys Thr Gln Pro Phe Leu
380 385 390

His Asp Gly Tyr Ala Val Ser Trp Asn Thr Leu Asp Val Asn Lys

395 400 405

Thr His Ile Ser Val Asn Gly Arg Lys Ile Arg Net Arg Cys Arg
410 415 420

Thr Ala Glu Asn Thr Pro Leu Pro Ile Ala Gly Val Leu Leu Pro
425 430 435

Thr Ile Pro Gly Lys Leu Asp Val Asn Lys Ser Lys Thr His Ile
440 445 450

Ser Val Asn Gly Arg Lys Ile arg Met Arg Cys Arg Ala Ile Asp
455 460 465

Gly Asp Val Thr Phe Cys Arg Pro Lys Ser Pro Val Tyr Val Gly
470 475 480

Asn Gly Val His Ala Asn Leu His Val Ala Phe His Arg Ser Ser
485 490 495

Ser Glu Lys Ile His Ser Asn Glu Ile Ser Ser Asp Ser Ile Gly
500 505 510

Val Leu Gly Tyr Gln Lys Thr Val Asp His Thr Lys Val Asn Ser
515 520 525

Lys Leu Ser Leu Phe Phe Glu Ile Lys Ser
530 535

What Is Claimed Is:

1. A cytotoxin which is site-specifically modified to include one unpaired cysteine residue located in relation to the cytokine's receptor-binding site such that said modification does not significantly affect the receptor-binding or the biological activity of the cytotoxin and such that conjugation of a binding molecule to the unpaired cysteine residue blocks receptor-binding.

2. The cytotoxin of claim 1 which is pseudomonas exotoxin or diphtheria toxin.

3. The cytotoxin of claim 2 wherein the unpaired cysteine residue is substituted for the lysine No. 20, serine No. 25, serine No. 88, serine No. 96, serine No. 158, arginine No. 182, serine No. 188, serine No. 192, lysine No. 223, or serine No. 245 residue of pseudomonas exotoxin.

4. The cytotoxin of claim 2 wherein the unpaired cysteine residue is substituted for one of the serine, tyrosine, asparagine, glutamine, threonine, lysine, histidine, arginine, aspartate, or glutamate residues located between amino acid residue numbers 405 to 613 of diphtheria toxin.

5. The cytotoxin of claim 1 conjugated to a binding molecule.

6. The conjugate of claim 5 wherein the binding molecule is a monoclonal antibody, an $F(ab')_2$ or Fab fragment, or a ligand which binds to a cell surface receptor.

7. The conjugate of claim 6 wherein the ligand is interleukin-2, interleukin-6, or a transforming growth factor which binds to a cell surface receptor.

8. An immunotoxin comprising the cytotoxin of claim 1 conjugated to a binding molecule via the unpaired cysteine residue, and wherein the conjugation is with a bifunctional

linking agent.

9. The immunotoxin of claim 8 wherein the linking agent is N-succinimidyl 3-(2-pyridylthio)propionate or 4-succinimidyloxycarbonyl-α-methyl-α-(2-pyridyldithio)toluene.

10. The immunotoxin of claim 7 wherein the binding molecule is a monoclonal antibody, an $F(ab')_2$ or Fab fragment, or a ligand which binds to a cell surface receptor.

INTERNATIONAL SEARCH REPORT

Inter...onal application No.
PCT/US93/00358

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :C07K 13/00
US CL :530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, Dialog, Intelligenetics

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CRC Critical Reviews in Therapeutic Drug Carrier Systems, Vol. 2, No. 4, issued 1986, D.M. Neville, Jr., "Immunotoxins: Current use and future prospects in bone marrow transplantation and cancer treatment", pages 329-352, see entire document.	1-10
Y	Cell, Vol. 47, issued 05 December 1986, I. Pastan et al., "Immunotoxins", pages 641-648, see entire document.	1-10
Y	US, A, 4,664,911 (Uhr et al) 12 May 1987, see entire document.	2-4, 8, 9
Y	Science, Vol. 254, issued 22 November 1991, I. Pastan et al, "Recombinant toxins for cancer treatment", pages 1173-1177, see entire document.	5-7, 10
Y	J. Immuno. Meth., Vol. 121, issued 1989, D.E. Myers et al., "The effects of aromatic and aliphatic maleimide crosslinkers on anti-CD5 ricin immunotoxins", pages 129-142, see entire document.	5-7, 10

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be part of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
02 March 1993	30 MAR 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer KAREN COCHRANE CARLSON, PH.D. Telephone No. (703) 308-0196

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